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Research Article

Peroxisome proliferator-activated receptor γ agonism attenuates endotoxaemia-induced muscle protein loss and lactate accumulation in rats

Hannah Crossland, Dumitru Constantin-Teodosiu, Sheila M. Gardiner and Paul L. Greenhaff

MRC/Arthritis Research UK, Centre for Musculoskeletal Ageing Research, [2]School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, U.K.

Correspondence: Paul L. Greenhaff (Paul.Greenhaff@nottingham.ac.uk)

The peroxisome proliferator-activated receptor γ (PPARγ) agonist rosiglitazone (Rosi) appears to provide protection against organ dysfunction during endotoxaemia. We examined the potential benefits of Rosi on skeletal muscle protein maintenance and carbohydrate metabolism during lipopolysaccharide (LPS)-induced endotoxaemia. Sprague-Dawley rats were fed either standard chow (control) or standard chow containing Rosi (8.5 ± 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) for 2 weeks before and during 24 h continuous intravenous infusion of LPS (15 $\mu g \cdot kg^{-1} \cdot h^{-1}$) or saline. Rosi blunted LPS-induced increases in muscle tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) mRNA by 70% ($P<0.05$) and 64% ($P<0.01$) respectively. Furthermore, Rosi suppressed the LPS-induced reduction in phosphorylated AKT and phosphorylated Forkhead box O (FOXO) 1 protein, as well as the up-regulation of muscle RING finger 1 (MuRF1; $P<0.01$) mRNA and the LPS-induced increase in 20S proteasome activity ($P<0.05$). Accordingly, LPS reduced the muscle protein:DNA ratio (~30%, $P<0.001$), which Rosi offset. Increased muscle pyruvate dehydrogenase kinase 4 (PDK4) mRNA ($P<0.001$) and muscle lactate accumulation ($P<0.001$) during endotoxaemia were suppressed by Rosi. Thus, pre-treatment with Rosi reduced muscle cytokine accumulation and blunted muscle protein loss and lactate accumulation during endotoxaemia, and at least in part by reducing activation of molecular events known to increase muscle protein breakdown and mitochondrial pyruvate use.

Introduction

Skeletal muscle wasting and the development of muscle lactic acidosis and insulin resistance are important clinical consequences of sepsis, and if sustained, can have a detrimental impact on patient recovery and survival. The rapid and marked loss of skeletal muscle mass during sepsis is thought to, in part, reflect the activation of several pathways important in muscle protein breakdown, including ubiquitin–proteasome pathway (UPP)-, calpain- and lysosome-dependent proteolytic pathways [1–4]. Sepsis also results in the inhibition of muscle carbohydrate oxidation, not least by increasing muscle pyruvate dehydrogenase kinase 4 (PDK4) abundance, which inhibits pyruvate dehydrogenase complex (PDC) activation [5–7], thereby decreasing PDC flux and increasing muscle lactate accumulation. Importantly, it has been demonstrated that pro-inflammatory cytokines, and in particular tumour necrosis factor-α (TNF-α), play a major role in the aetiology of muscle atrophy [8] and dysregulation of muscle carbohydrate metabolism [9]. Thus, suppressing the production of pro-inflammatory cytokines during sepsis may prove beneficial in reducing the loss of muscle mass, maintaining carbohydrate oxidation and reducing lactic acidosis associated with this condition.

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Increasing evidence suggests that activation (dephosphorylation) of the Forkhead box O (FOXO) family of transcription factors, through suppression of AKT signalling, plays an important part in triggering muscle atrophy, partly via the transcriptional up-regulation of muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), which are key mediators of UPP-dependent skeletal muscle proteolysis [10–12]. FOXO transcription factors may also play an important role in the inhibition of muscle carbohydrate oxidation in catabolic conditions, through up-regulation of PDK4 transcription and consequently decreased PDC activation and flux [13,14]. Indeed, we have provided substantive evidence supporting a role for cytokine-mediated suppression of the AKT/FOXO signalling pathway in the simultaneous induction of muscle atrophy and impairment of muscle carbohydrate oxidation during lipopolysaccharide (LPS)-induced endotoxaemia in rodents [15,16].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [17]. Chronic administration of the potent and selective PPARγ agonist rosiglitazone (Rosi; [18]) has been reported to improve insulin sensitivity and suppress UPP-dependent muscle protein breakdown in a leptin receptor-deficient mouse model (*db/db*) of dyslipidaemia-mediated insulin resistance [19], which was attributed to the preservation of AKT/FOXO signalling in *db/db* mice [19]. PPARγ agonists may also be useful in the treatment of inflammatory conditions. For example, in animal models of endotoxaemia, treatment with PPARγ activators has been shown to reduce circulating TNF-α and IL-1β in LPS-treated mice [20], and also to have protective effects on multiple organs following LPS treatment by unresolved mechanisms [21–23]. However, the potential beneficial effects of PPARγ agonists on maintenance of skeletal muscle metabolic function during endotoxaemia, and in particular, their impact on skeletal muscle atrophy and/or carbohydrate metabolism during endotoxaemia, have not been reported.

In the present study, therefore, we hypothesized that administration of Rosi to rodents for 2 weeks before and during endotoxaemia would reduce LPS-induced skeletal muscle cytokine mRNA abundance. As a direct consequence of this, we anticipated cytokine-mediated up-regulation of several molecular events known to increase muscle protein breakdown and lactate accumulation and reduce carbohydrate oxidation would be supressed. Accordingly, we proposed Rosi administration would blunt the induction of muscle protein loss and dysregulation of oxidative carbohydrate use that we have previously observed in skeletal muscle during endotoxaemia [15].

Experimental

Ethical approval

All procedures were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project and Personal Licence authority (Animal Scientific Procedures Act 1986).

Animals

Male Sprague-Dawley rats (350–450 g) were purchased from Charles River (Margate, U.K.) and housed in the Biomedical Services Unit at the University of Nottingham, with free access to food and water. Room temperatures were maintained at 21 ± 2°C, and lights were on from 06.00 to 18.00 h.

Experimental design, surgical preparation and tissue collection

Animals were divided into two groups: one group (n=16) was fed a standard rat chow (Teklad Global 18% protein rodent diet, Bicester, Oxon, U.K.) supplemented with Rosi (0.1 mg · g^{-1} food; Bepharm Ltd., China) for 14 days (and throughout subsequent experiments). A control ('Con') group (n=16) received standard rat chow. During the 14-day period, the rats were housed in pairs, with daily food intake measured twice weekly. Based upon the daily food intake, the average intake of Rosi was calculated to be 8.5 ± 0.1 mg · kg^{-1} · day^{-1}. The dose of Rosi to be administered was chosen on the basis of the work of Wang et al. [19], who demonstrated improvements in muscle AKT signalling in a mouse model of insulin resistance when Rosi was administered chronically at a dose of 8 mg · kg^{-1} · day^{-1} for 35 days.

After 14 days, animals were anaesthetized (fentanyl citrate (Janssen-Cilag, High Wycombe, U.K.) and medetomidine (Domitor; Pfizer, Sandwich, UK; 300 μg · kg^{-1} of each i.p.)) and intravenous catheters were implanted in the right jugular vein. Anaesthetic reversal and the provision of analgesia were achieved with s.c. atipamezole (Antisedan; Pfizer, Sandwich, U.K.; 1 mg · kg^{-1}) and buprenorphine (Vetergesic; Alstoe Animal Health, York, U.K.; 0.03 mg · kg^{-1}), and animals were returned to individual home cages. The catheters were connected to fluid-filled swivels for overnight infusion of heparinized saline (15 U · ml^{-1}, 0.4 ml · h^{-1}) to maintain catheter patency.

Experiments began 24 h after catheterization. Eight of the prepared animals in each of the two diet groups were subjected to a continuous 24 h infusion of LPS (15 μg · kg^{-1} · h^{-1}; *Escherichia coli* serotype 0127: B8; Sigma–Aldrich,

Poole, U.K.). The remaining animals in each diet group (n=8 per group) received an equal volume of saline ('Sal'; 0.4 ml · h^{-1}) for 24 h. After 24 h, animals were terminally anaesthetized with thiobutabarbital sodium (Inactin; Sigma–Aldrich, St. Louis, U.S.A.; 80 mg · kg^{-1} i.v.), after which the extensor digitorum longus (EDL) muscle from both hindlimbs was removed and immediately snap-frozen in liquid nitrogen. The EDL muscle was selected due to its high susceptibility to atrophy during endotoxaemia [15,24].

Muscle protein and DNA measurements

The muscle protein:DNA ratio was used to assess changes in muscle protein mass, as it has previously been shown that reduced skeletal muscle protein content is a consistent feature of critically ill patients [25,26], in the face of substantial increased extracellular muscle water content. These findings strongly suggest that under catabolic conditions where substantial changes in tissue fluid may occur, the protein:DNA ratio may be a more reliable measure of changes in muscle protein mass than other approaches such as cross-sectional area or muscle weight.

A portion of frozen EDL muscle was freeze-dried and powdered, and alkaline-soluble protein and DNA were extracted from 2 to 3 mg powdered muscle according to Forsberg et al. [27]. Briefly, powdered muscle was extracted in 0.2 mol · l^{-1} perchloric acid (PCA), before being centrifuged at 3000 ***g*** for 15 min. The muscle residue was dissolved in 0.3 mol · l^{-1} KOH and samples were assessed for alkaline-soluble protein, using the Lowry method [28]. In the same extract, DNA was hydrolyzed by incubating in 1 mol · l^{-1} PCA at 70°C and the digest was used to measure DNA using a modification of the diphenylamine method [27].

Real-time PCR measurements

Total RNA was isolated from frozen wet EDL muscle (20–30 mg) using Tri Reagent (Sigma–Aldrich, Poole, U.K.), according to the manufacturer's protocol. RNA was quantified through spectrophotometric measurement at 260 and 280 nm, and reverse transcription was carried out in a reaction comprising of 1 μg RNA, random primers, MMLV reverse transcriptase, dNTPs and RNase inhibitor (all from Promega, Southampton, U.K.). Resulting cDNA was diluted 4-fold and used in real-time PCR reactions in an ABI Prism 7700 Sequence Detection system (Applied Biosystems) using Assay-on-Demand Taqman® primer/probe sets (PPARγ, TNF-α, interleukin-6 (IL-6), FOXO1, MAFbx, MuRF1, PDK4 and cathepsin-L) from Applied Biosystems (Foster City, U.S.A.). The housekeeping gene hydroxymethylbilane synthase (HMBS) was used as an internal control as it was unaffected by the treatments (data not shown).

Relative changes in mRNA expression between groups were calculated using the $2^{-\Delta\Delta C}{}_t$ method, normalized against HMBS. A calibrator in the Con/Sal group was given a value of 1, and fold changes in mRNA expression for the Con/LPS-treated, Rosi/Sal-treated and Rosi/LPS-treated groups were calculated, relative to the calibrator.

Protein extraction and Western blotting measurements

Proteins were extracted from 20 to 30 mg frozen wet tissue by homogenization in a buffer consisting of 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF and complete protease inhibitor tablet (Roche, West Sussex, U.K.). Extracts were centrifuged at 13000 ***g*** for 10 min at 4°C to collect the supernatant. Protein samples (10 μg) were run on Criterion XT 4–12% Bis-Tris gels (Bio-Rad, Hemel Hempstead, U.K.) at 200 V for 1 h, and transferred to PVDF membranes for 1 h at 100 V. Membranes were blocked in 2.5% BSA diluted in Tris-buffered saline (TBS) and 0.1% Tween-20 and incubated with primary antibodies (phosphorylated AKT (Ser^{473}; pAKT, #4058), phosphorylated FOXO1 (Ser^{256}; pFOXO1, #9461) phosphorylated FOXO3 (Ser^{253}; pFOXO3, #9466), total AKT (#4691), total FOXO1 (#2880) and total FOXO3 (#12829); all from Cell Signaling Technology, Danvers, U.S.A.) diluted 1:2000 overnight at 4°C. Membranes were washed 3 × 5 min with TBS-Tween, incubated with peroxidase-conjugated anti-rabbit secondary antibody (New England Biolabs, Hitchin, U.K.) 1:2000 for 1 h at room temperature, and incubated with enhanced chemiluminescence detection reagent (Millipore, Watford, U.K.). Bands were visualized using a Chemidoc XRS system (Bio-Rad, Hemel Hempstead, U.K.), and were quantified by measurement of peak density and normalization against Coomassie Brilliant Blue staining of the membrane.

Muscle metabolite measurements

Freeze-dried and powdered muscle samples (5–10 mg) were extracted using 0.5 mol · l^{-1} PCA containing 1 mmol · l^{-1} EDTA, then neutralized with 2.1 mol · l^{-1} $KHCO_3$. Muscle lactate concentration was determined using a modification of the spectrophotometric method of Harris et al. [29].

Clinical Science (2017) **131** 1437–1447
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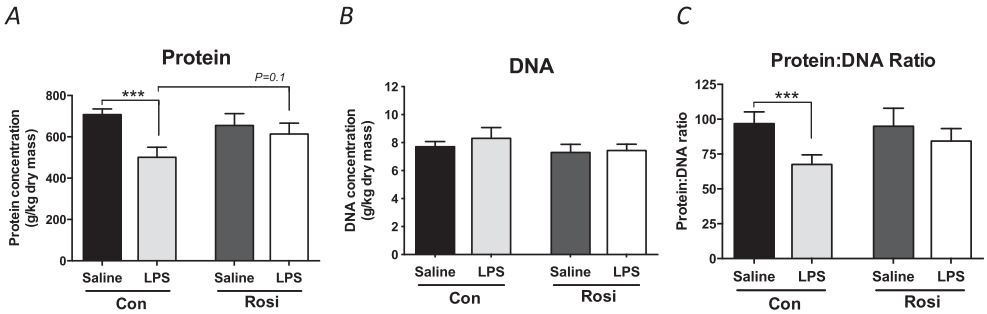

Figure 1. Effect of Rosi on alkaline-soluble protein (A), DNA (B) and the protein to DNA ratio (C) in EDL muscle following 24-h LPS or saline (Sal) infusion

Values represent mean + SEM and n=8 for each group. Significantly different from Con/Sal: ***P<0.001.

Muscle enzyme activity measurements

Frozen muscle (15–25 mg) was homogenized, and the soluble muscle extract was used to measure the chymotrypsin-like activity of the 20S proteasome using the fluorogenic substrate N-Suc-Leu-Leu-Val-Tyr-7-Amido-4-Methyl-Coumarin (Sigma–Aldrich, Poole, U.K.), according to Dawson et al. [30]. The same muscle homogenate was also used to determine cathepsin-L activity using the substrate Z-Phe-Arg-7-Amido-4-Methyl-Coumarin (Sigma–Aldrich, Poole, U.K.), according to Bergmeyer [31].

Statistical analysis

Data are presented as mean ± SEM. Comparisons between treatment groups were performed using one-way ANOVA, with the Fisher's least significant difference (LSD) *post hoc* test being used to locate any significant differences. $P<0.05$ was taken as statistically significant.

Results

The average body mass of rats fed the diet supplemented with Rosi increased by 100 ± 8 g during the 14-day period, which was no different to the increase of 107 ± 7 g observed in animals fed the Con diet. Average food intake during this period was no different between treatment groups, and was calculated to be 85.0 ± 1.4 $g \cdot kg^{-1} \cdot day^{-1}$ in animals fed the Con diet compared with 78.0 ± 1.0 $g \cdot kg^{-1} \cdot day^{-1}$ in rats fed the Rosi supplemented diet.

Treatment with Rosi for 14 days had no effect on any of the measured variables in saline-infused animals (with the exception of muscle PPARγ mRNA expression; see below). Thus, there were no significant differences between any of the measurements made, i.e. in terms of muscle protein catabolism, cytokine abundance and AKT/FOXO signalling pathways, when comparing animals that received the Con diet plus saline-infusion (Con/Sal) versus Con diet with Rosi plus saline-infusion (Rosi/Sal; Figures 1–5).

Protein:DNA ratio

Compared with Con/Sal, muscle alkaline-soluble protein and the protein:DNA ratio were ∼30% lower in Con plus LPS-treated (Con/LPS) animals (both $P<0.001$; Figure 1A and C), indicating that LPS infusion exerted a significant catabolic effect on EDL muscle. DNA content was no different across treatment groups (Figure 1B). In Rosi-treated rats infused with LPS (Rosi/LPS), the protein:DNA ratio was not different from that in Rosi/Sal-treated animals; however, there was also no significant difference between the protein:DNA ratio in Con/LPS and Rosi/LPS-treated animals (but there was a trend for the alkaline-soluble protein content to be lower in Con/LPS, P=0.1; Figure 1A). These findings indicate that Rosi administration was able to suppress the loss of muscle protein associated with endotoxaemia.

Muscle mRNA expression

Differences in muscle mRNA expression in Con/LPS-, Rosi/Sal- and Rosi/LPS-treated animals relative to Con/Sal-treated animals are shown in Figure 2. PPARγ mRNA expression was greater in Con/LPS-, Rosi/Sal- and Rosi/LPS-treated animals compared with Con/Sal-treated animals. LPS infusion in rats fed the control diet resulted in markedly greater mRNA expression of TNF-α (13-fold, $P<0.01$), IL-6 (14-fold, $P<0.001$), FOXO1 (3.9-fold, $P<0.001$), MAFbx (4.8-fold, $P<0.01$), MuRF1 (17-fold, $P<0.001$), cathepsin-L (16-fold, $P<0.001$) and

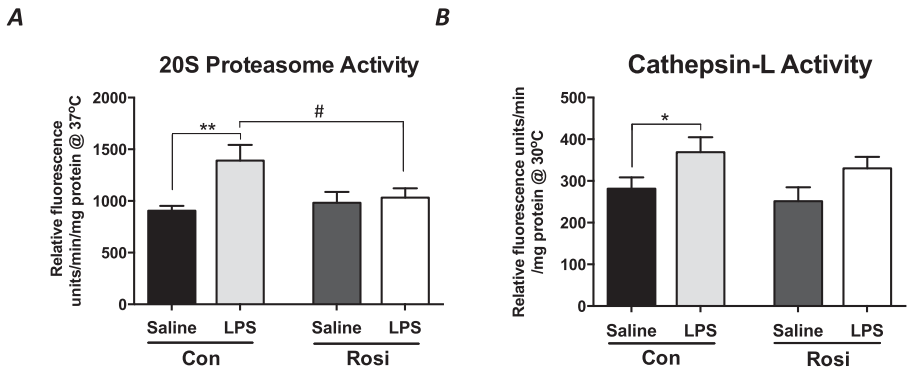

Figure 5. Effect of Rosi on 20S proteasome (A) and cathepsin-L (B) activity in rat EDL muscle following 24 h of LPS or saline (Sal) infusion

Values are mean and vertical bars represent SEM and n=8 for each group. Significantly different from Con/Sal group: $^{*}P<0.05$; $^{**}P<0.01$. Significantly different from Con/LPS group: $^{\#}P<0.05$.

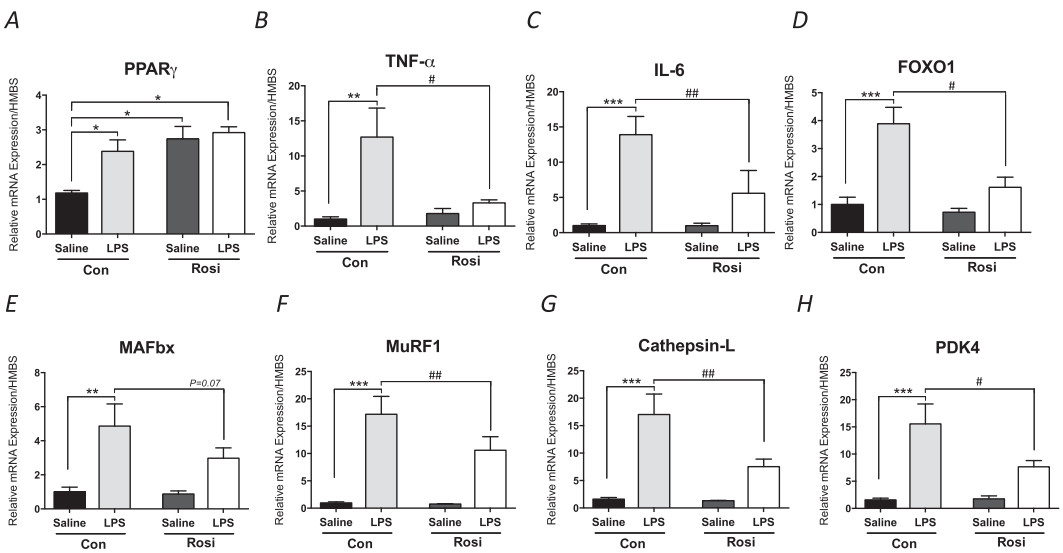

Figure 2. Effect of Rosi on the mRNA expression of (A) PPARγ, (B) TNF-α, (C) IL-6, (D) FOXO1, (E) MAFbx, (F) MuRF1, (G) cathepsin-L and (H) PDK4 in rat EDL muscle following 24-h LPS or saline (Sal) infusion

Relative mRNA expression of saline controls was set at 1. Values are mean and vertical bars represent SEM and n=8 for each group. Significantly different from Con/Sal: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Significantly different from Con/LPS group: $^{\#}P<0.05$; $^{\#\#}P<0.01$.

PDK4 (15-fold, $P<0.001$) relative to Con/Sal-treated animals. These findings are consistent with previous observations during endotoxaemia in EDL muscle [15,16]. Rosi treatment markedly supressed the LPS-induced increases in mRNA expression for all genes analysed, with the exception of MAFbx, where the difference did not reach statistical significance (P=0.07 versus Con/LPS; Figure 2E). Thus, Rosi administration significantly suppressed LPS-induced increases in mRNA expression of genes linked to inflammatory signalling and catabolic processes.

Muscle protein expression

Infusion of LPS was associated with significantly less (∼25%) muscle phosphorylated AKT (Ser^{473}) expression relative to Con/Sal-treated animals ($P<0.05$; Figure 3A), consistent with a suppression of AKT signalling, whereas there was no significant difference in phosphorylated AKT expression between Rosi/LPS-treated animals and Rosi/Sal-treated animals. Expression of total AKT was unaffected by treatments (Figure 3B). Similarly, phosphorylated FOXO1 (Ser^{256}) and FOXO3 (Ser^{253}) protein expression was less in Con/LPS relative to Con/Sal-treated animals (∼45%, $P<0.001$, Figure 3C; ∼45%, $P<0.001$, Figure 3E respectively). There was no difference in FOXO1 (Ser^{256}) expression levels between Rosi/Sal- and Rosi/LPS-treated animals (Figure 3C), but in the case for FOXO3 (Ser^{253}) protein expression

Clinical Science (2017) **131** 1437–1447
DOI: 10.1042/CS20170958

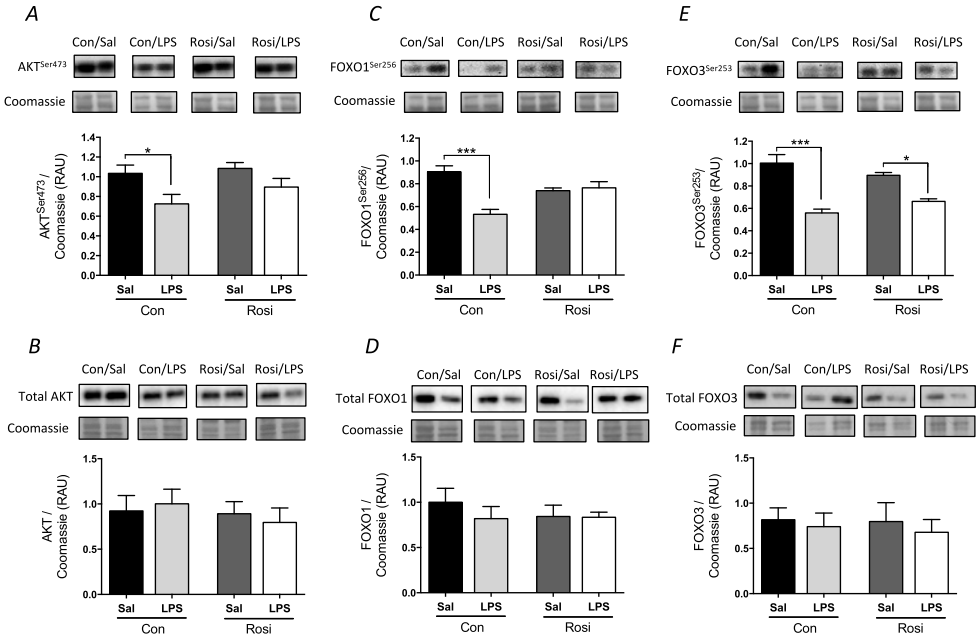

Figure 3. Effect of Rosi on phosphorylation of AKT^{Ser473} (A), total AKT (B), FOXO1^{Ser256} (C), total FOXO1 (D), FOXO3^{Ser253} (E) and total FOXO3 (F) in rat EDL muscle following 24-h LPS or saline (Sal) infusion

Values represent mean + SEM and n=8 for each group. Representative blots for each protein measured are shown for 2 of 8 animals in each experimental group. Significantly different from Con/Sal or Rosi/Sal: *P<0.05; ***P<0.001.

was less in Rosi/LPS relative to Rosi/Sal-treated animals (∼20%, P<0.05, Figure 3E). Thus, Rosi appeared to prevent the LPS-induced reduction in FOXO1 but not FOXO3 phosphorylation. There was also no difference in AKT (Ser^{473}) expression and phosphorylated FOXO1 (Ser^{256}) expression when comparing Con/LPS- and Rosi/LPS-treated animals (Figure 3A and C). Finally, there were no differences in total FOXO1 (Figure 3D) or total FOXO3 (Figure 3F) across treatment groups.

Muscle lactate and enzyme activities

Muscle lactate concentration was 2-fold greater in Con/LPS compared with Con/Sal-treated animals (P<0.001; Figure 4), whereas it was not significantly greater in Rosi/LPS versus Rosi/Sal-treated animals. There was also a trend for muscle lactate concentration to be lower in Rosi/LPS compared with Con/LPS-treated animals (P=0.08; Figure 4).

Muscle 20S proteasome activity was 1.5-fold greater in Con/LPS-treated animals compared with Con/Sal-treated animals, while 20S proteasome activity was no different between Rosi/Sal- and Rosi/LPS-treated animals (P<0.01; Figure 5A), indicating that the LPS-associated increases in UPP-dependent muscle protein breakdown were suppressed by Rosi. Furthermore, 20S proteasome activity was significantly less in Rosi/LPS versus Con/LPS-treated animals (P<0.05; Figure 5A). Muscle cathepsin-L activity was significantly greater in Con/LPS-treated rats compared with Con/Sal (1.3-fold, P<0.05; Figure 5B), whereas cathepsin-L activity was no different between Rosi/LPS- and Rosi/Con-treated animals. However, cathepsin-L activity in Con/LPS-treated rats was not different from Rosi/LPS-treated animals (Figure 5B).

Discussion

To our knowledge, this is the first study to demonstrate clear protective effects of PPARγ agonism on muscle protein loss and lactate accumulation during *in vivo* LPS-induced endotoxaemia. It also provides mechanistic insight of the molecular events underpinning this protection. We hypothesized that dietary administration of the PPARγ agonist Rosi to rodents for 2 weeks before and during 24 h of LPS-induced endotoxaemia would reduce skeletal muscle cytokine mRNA abundance that we have previously observed in skeletal muscle during endotoxaemia [15]. We hypothesized further that as a direct consequence of this, cytokine provoked up-regulation of several molecular events known to increase muscle protein breakdown and lactate accumulation, and reduce carbohydrate oxidation, would be suppressed. More specifically, we speculated that Rosi administration would blunt LPS-mediated

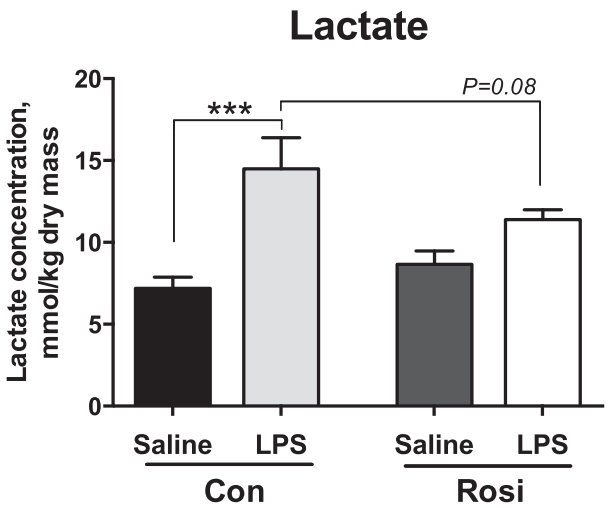

Figure 4. Lactate concentrations in rat EDL muscle

Lactate content in EDL muscle from rats administered with LPS or saline (Sal) for 24 h, following Rosi treatment. Values are mean and vertical bars represent SEM and n=8 for each group. Significantly different from Con/Sal group: ***P<0.001.

down-regulation of muscle AKT signalling, activation of FOXO transcription factors and up-regulation of downstream FOXO targets thought to mediate proteolysis (MAFbx, MuRF1 and cathepsin-L) and limit mitochondrial pyruvate oxidation (PDK4). In keeping with this, our results convincingly demonstrate that Rosi treatment exerted a significant anti-inflammatory effect in muscle during endotoxaemia (Figure 2B and C), which was associated with preservation of phosphorylation of AKT (activation, Figure 3A) and FOXO1 (inactivation, Figure 3B) and markedly reduced mRNA expression of key factors involved in muscle protein breakdown (i.e. FOXO1, MuRF1, cathepsin-L; Figure 2D, F and G respectively). This coincided with the suppression of 20S proteasome activity in Rosi/LPS-treated animals (Figure 5A). Moreover, treatment with Rosi appeared to offset the LPS-induced reduction in the muscle protein:DNA ratio (Figure 1C). The findings that PDK4 mRNA expression (Figure 2H) and muscle lactate concentration (Figure 4) were lower in Rosi/LPS-treated rats strongly suggest that Rosi also had a beneficial effect on mitochondrial pyruvate utilization. Collectively, these results are consistent with the involvement of cytokine-mediated modulation of muscle AKT/FOXO1-dependent signalling during endotoxaemia, which can be supressed by PPARγ agonism, thereby reducing muscle atrophy and maintaining mitochondrial pyruvate use.

Previous rodent studies have reported protective effects of PPARγ agonism in organ damage caused by endotoxaemia [21–23], which were exerted by both receptor-mediated actions and anti-inflammatory properties of PPARγ agonists [32]. The markedly lower expression of muscle TNF-α and IL-6 mRNA in Rosi/LPS-treated animals (Figure 2B and C), along with the preservation of the muscle protein:DNA ratio in these same animals (Figure 1C; which is a reliable marker for changes in muscle protein mass, particularly during sepsis where significant changes in tissue fluid content occur [25]), suggests that the protective effects of PPARγ agonism was due to an anti-inflammatory influence. Furthermore, the finding that Rosi/Sal and Rosi/LPS treatments elicited similar increases in muscle PPARγ mRNA expression (Figure 2A) supports this suggestion, and also indicates the protective effects exerted by Rosi may not have been PPARγ-dependent per se. Inclusion of a PPARγ antagonist to test whether the effects observed by Rosi were directly dependent on PPARγ activation would help to confirm these results. We have previously shown that suppressing the increase in muscle pro-inflammatory cytokine mRNA expression during 24-h LPS infusion by concurrently infusing low-dose dexamethasone (Dex), a potent synthetic glucocorticoid, also preserved the muscle protein:DNA ratio [16], lending further support to our assertion. Given LPS has been shown to down-regulate PPARγ mRNA and protein expression *in vivo* in hepatic tissue and *in vitro* in Kupffer cells and HepG2 cells via both TNF-α- [33] and IL6-mediated [34] mechanisms, the elevation in PPARγ mRNA expression in the skeletal muscle of Con/LPS-treated animals above Con/Sal-treated animals in the present study was unexpected and is not easily explained.

Down-regulation of AKT signalling in inflammatory catabolic conditions is associated with dephosphorylation (activation) of FOXO transcription factors and increased mRNA expression of FOXO downstream gene targets [12,19], which we have confirmed in skeletal muscle in the same rat model of LPS-induced endotoxaemia as used in the present study [15]. Furthermore, we have demonstrated in this same rodent model of clinical endotoxaemia

Clinical Science (2017) **131** 1437–1447
DOI: 10.1042/CS20170958

that suppression of LPS-induced muscle cytokine elevation achieved by concurrent low dose Dex infusion preserves phosphorylation of AKT (activation) and FOXO1 (inhibition), reduces abundance and activation of their downstream targets and maintains muscle protein content [16]. Since PPARγ agonists are known to be anti-inflammatory [32] and chronic administration of Rosi has been demonstrated to prevent the down-regulation of AKT signalling, activation of FOXO1 and muscle protein breakdown in a genetic mouse model (*db/db*) of dyslipidaemia-mediated insulin resistance [19], we aimed to determine whether Rosi administration would result in similar positive events during the acute catabolic state of LPS-induced endotoxaemia and thereby attenuate the muscle protein loss that occurs. Consistent with the observations of Wang et al. [19], administration of Rosi for 14 days prior to and during LPS-induced endotoxaemia appeared to, at least partially, prevent the LPS-induced reduction in phosphorylated or 'active' AKT protein, since there was no significant difference in phosphorylated AKT in muscles of Rosi/LPS-treated animals compared with the Rosi/Sal group (Figure 3A). Also in line with the findings of Wang et al. [19], the LPS-induced increase in FOXO1 mRNA expression was significantly reduced by Rosi treatment (Figure 2D), and the LPS-mediated reduction in cytosolic FOXO1 (protein) dephosphorylation was prevented by Rosi administration (Figure 3C). Collectively, these events were associated with the preservation of muscle protein content (Figure 1), but Rosi did not appear to prevent the LPS-induced reduction in FOXO3 phosphorylation (Figure 3E). It is not completely clear why Rosi inhibited LPS-induced reductions in FOXO1, but not FOXO3 phosphorylation. It is feasible that their phosphorylation sites may be regulated by different upstream proteins, or that FOXO1 was more responsive to the Rosi-mediated reduction in cytokine expression levels than FOXO3. Given phosphorylation (inactivation) of FOXO transcription factors occurs on three conserved residues, and in the present study we determined FOXO1 Ser^{256} and FOXO3 Ser^{253}, we can speculate that Rosi may have attenuated LPS-induced dephosphorylation on the Ser^{256} site, but not on the Ser^{253} site, thereby explaining our observation.

Since increased activity of the UPP is thought to significantly contribute to muscle protein loss during sepsis [1,35,36], the greater mRNA expression of MAFbx and MuRF1 and activity of the 20S proteasome observed with Con/LPS treatment in the present study (Figures 2E, 2F and 5A), as seen previously with this model [15,16], clearly point to increased UPP activity being, at least partly, responsible for the reduced muscle protein:DNA ratio observed in these animals. More importantly, however, Rosi treatment significantly dampened the LPS-induced increases in muscle MuRF1 mRNA (Figure 2F), tended to reduce the increase in MAFbx mRNA (Figure 2E) and significantly diminished muscle 20S proteasome activity (Figure 5A). Collectively, these findings convincingly indicate that Rosi was able to suppress muscle UPP-dependent protein breakdown during endotoxaemia, and at least in part by diminishing the cytokine-mediated burden on AKT and FOXO1 signalling. It should be acknowledged however that nuclear factor kappa B (NF-κB) and p39 mitogen-activated protein kinase (MAPK) signalling pathways have also been implicated in the cytokine-dependent up-regulation of the proteasome system [37,38].

Evidence also exists supporting a role for FOXO family transcription factors in the up-regulation of muscle autophagy and lysosomal proteolysis [39–41]. Indeed, cathepsin-L, a lysosomal protease that is up-regulated in a variety of models of muscle atrophy [42,43], and most likely by a cytokine mediated mechanism [44], is a FOXO1 downstream gene target in skeletal muscle [45]. We have previously shown cathepsin-L to be up-regulated at both the mRNA and protein level in endotoxaemia [16], which was dampened by Dex-mediated suppression of LPS-induced muscle cytokine elevation [16]. In the present study, Con/LPS treatment markedly increased cathepsin-L mRNA expression, which was robustly blunted in Rosi/LPS-treated animals, presumably also due to the anti-inflammatory properties of Rosi (Figure 2G). Furthermore, whilst muscle cathepsin-L activity was greater in Con/LPS-treated animals compared with Con/Sal-treated animals, activity was no different between Rosi/LPS-treated and Rosi/Sal-treated animals (it is acknowledged that cathepsin-L activity was also no different between Con/LPS- and Rosi/LPS-treated animals; Figure 5B). Therefore, based upon cathepsin-L mRNA expression and protein activity measurements, it would appear that Rosi had some capacity to blunt an LPS-induced increase in cathepsin-L-mediated muscle protein breakdown, thereby contributing to the preservation of muscle protein content observed, and presumably also by dampening of cytokine-mediated dysregulation of AKT/FOXO1 signalling in endotoxaemia.

Increased muscle PDK4 expression can lead to a down-regulation of muscle mitochondrial pyruvate oxidation, through its ability to phosphorylate and thereby inactivate the mitochondrial PDC [46]. We have previously demonstrated a cytokine-linked increase in PDK4 mRNA and protein expression, coupled to the inhibition of PDC activity and stimulation of lactate accumulation in muscle during LPS-induced endotoxaemia in this rodent model [7,15]. Furthermore, we have strongly implicated the dysregulation of AKT/FOXO signalling in these events [15]. The up-regulation of muscle PDK4 mRNA observed with Con/LPS treatment in the present study (Figure 2H), along with the increase in muscle lactate accumulation (Figure 4), is entirely consistent with our previous observations [7,15]. Moreover, we have also reported that blunting the cytokine response to LPS using low-dose Dex offset the dysregulation of Akt signalling, and normalized muscle PDK4 mRNA and protein expression, PDC activity and lactate

accumulation [16]. Directly in keeping with this, the Rosi-mediated normalization of the muscle cytokine response observed during Con/LPS treatment of the present study (Figure 2B and C) was associated with the suppression of LPS-induced up-regulation of PDK4 mRNA expression (by 50%, Figure 2H), and the normalization of muscle lactate accumulation (Figure 4) clearly suggesting Rosi administration maintained mitochondrial pyruvate utilization during under these conditions.

In summary, this is the first study to demonstrate clear positive effects of PPARγ agonism on muscle protein loss and lactate accumulation during *in vivo* LPS-induced endotoxaemia. Dietary administration of the PPARγ agonist, Rosi, to rodents reduced skeletal muscle cytokine mRNA expression during *in vivo* LPS-induced endotoxaemia and dampened the LPS-mediated down-regulation of muscle AKT signalling, activation of FOXO1 transcription factor, and up-regulation of FOXO downstream target genes thought to mediate proteolysis (MAFbx, MuRF1 and cathepsin-L) and limit mitochondrial pyruvate oxidation (PDK4). This coincided with the suppression of 20S proteasome, and to a lesser extent cathepsin-L, activity in Rosi/LPS-treated animals. The findings that PDK4 mRNA expression and muscle lactate concentration were lower in Rosi/LPS-treated rats strongly suggest that Rosi had a beneficial effect on mitochondrial pyruvate utilization. Collectively, these results, along with our previous demonstration that low-dose Dex elicits similar events during LPS-induced endotoxaemia [16], are entirely consistent with cytokine-mediated modulation of AKT/FOXO1-dependent signalling in muscle during endotoxaemia, which if supressed (by either PPARγ agonism or glucocorticoid administration) can offset muscle net protein loss (atrophy) and maintain mitochondrial pyruvate oxidation during endotoxaemia, and most likely by a common AKT/FOXO1-dependent mechanism. The findings of the present study, therefore, contribute further insight towards a better understanding of the mechanisms regulating muscle atrophy and carbohydrate oxidation during endotoxaemia.

Clinical Perspectives

- Skeletal muscle wasting and muscle lactic acidosis are two important clinical consequences of sepsis, that if prolonged, can have a detrimental impact on patient recovery and survival. We aimed to determine the potential beneficial effects of PPARγ agonists on skeletal muscle atrophy and/or carbohydrate metabolism during endotoxaemia in rats.

- Pre-treatment with the PPARγ agonist Rosi reduced muscle cytokine mRNA and alleviated muscle protein loss and lactate accumulation during endotoxaemia, at least in part by reducing activation of molecular events linked to increased muscle protein breakdown and mitochondrial pyruvate use.

- We have demonstrated clear positive effects of Rosi on muscle wasting and lactate accumulation during endotoxaemia *in vivo*, contributing further insight into our understanding of the mechanisms underlying the regulation of muscle atrophy and mitochondrial pyruvate use during endotoxaemia.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

HC generated the experimental hypothesis, study design and research data, and wrote and edited the manuscript. DC-T and SMG generated the experimental hypothesis, study design and research data, and edited the manuscript. PLG generated the experimental hypothesis, study design, and wrote and edited the manuscript.

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Abbreviations

AKT, protein kinase B; EDL, extensor digitorum longus; FOXO, Forkhead box O family; HMBS, hydroxymethylbilane synthase; IL-6, interleukin-6; LPS, lipopolysaccharide; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1; PDC, pyruvate dehydrogenase complex; PDK4, pyruvate dehydrogenase kinase 4; PPAR, peroxisome proliferator-activated receptor; Rosi, rosiglitazone; TNF-α, tumour necrosis factor-α; UPP, ubiquitin–proteasome pathway.

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